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13. ABSTRACT (Maximum 200) This project is aimed at establishing bulk cultures of individual epithelial and stromal cells from mastectomy specimens of patients who are carrying genes that predispose them to breast cancer. Epithelial and stromal cells are being conditionally immortalized using a novel amphotropic retroviral vector transducing the tsA58-U19 SV40-derived construct. These cells are from patients who are mutation carriers of BRCA1, BRCA2, and p53. Samples have been received from 38 women, eight of which have a Li-Fraumeni - like phenotype, four with confirmed mutations. Other cases are presumptive carriers of BRCA1, BRCA2 or other mutations. In two cases the mutations have been identified and the others will be screened in the next year. In the majority of cases primary cultures have been established, cells banked and immortalization commenced. In parallel a detailed data-base is being established of <i>in situ</i> disease from patients that have been sent to the Royal Marsden Hospital Breast Unit since 1970. Over 5,500 slides have been reviewed of <i>in situ</i> breast cancer from over 700 cases. Material from over 500 patients has been selected for further study. The cases from 1994-96, with short follow up are being used to pilot the molecular studies. Sections have been cut and studies commenced on the molecular and immunocytochemical analyses of these lesions.				
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FOREWORD

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(5) INTRODUCTION:

Breast cancer is now recognised as an heterogeneous disease in which there are multiple molecular abnormalities which progressively accumulate to result in the clinical and morphological phenotypes seen as breast cancer. As the dissection of these molecular events is undertaken at the gene level it is essential that relevant cell systems are established to act as future targets in which to understand the function of the proteins encoded by these genes. In particular it will be necessary to establish human models in which to study the function of predisposing genes. Also it is essential that systems are established now that will enable researchers to study the importance of combinations of molecular genetic abnormalities and their relative contributions to the tumor phenotype. In parallel it is important that material is available from the earliest stages of malignancy which can be used to assess the relevance of the *in vitro* and molecular data.

This project is in two parts, which is focussed on developing an infrastructure resource that will enable research groups to address questions particularly related to the early stages of breast cancer evolution, and also to provide systems that will enable advances to be made related to prevention, diagnosis and treatment.

(I) Part 1 Familial Breast Cancer:

Between 5-10% of breast cancer is due to cancer predisposing genes. In the United Kingdom, there are 25,000 new cases of breast cancer per year, therefore about 2,500 cases each year could be due to a cancer predisposition gene. Two genetic models could account for the genetic predisposition to breast cancer. The first is the presence of rare, but highly penetrant genes which would account for about 10% of all breast cancer cases; the second is more common, less penetrant genes which would confer a lower cancer risk to each individual gene carrier, but due to its wider distribution, such a gene would contribute to a larger number of breast cancer cases (maybe as high as 86%). It is now clear that familial breast cancer is a heterogeneous disease, and a combination of these two models is the most likely.

Two autosomal genes BRCA1, and BRCA2 with high penetrance have been cloned (1,2) and gene carriers have a lifetime risk of breast cancer of 80%. Although rare, germline mutations in the p53 gene confer a very high breast cancer risk - 90% by age 50, (3). It is likely that lower penetrance genes contribute to a larger percentage of overall population breast cancer risk. One such candidate would be ataxia telangiectasia (AT) since AT heterozygotes have a relative risk of breast cancer at about six times that of the general population (4). The AT gene has been recently cloned (5).

This project is aimed at providing a resource of cells, cell lines and frozen tissues from patients that have an increased risk of developing breast cancer due to the fact that they are carriers of breast cancer susceptibility genes. Included in the study are patients from families with BRCA1, BRCA2, Li-Fraumeni and Li-Fraumeni syndromes and patients with ataxia telangiectasia and Cowden's. Establishing these cells in culture will provide systems for both primary studies of the abnormal genes in comparison with the wild type, but also models in which to study synergistic effects of genes, so enabling analyses of the early events in breast cancer.

Such *in vitro* systems will also provide relevant models to:

- a) explore the reversal of the predisposed phenotype using genetic manipulation;
- b) carry out drug testing for both prevention and treatment;
- c) test radiation sensitivity to enhanced risk.

In order to study the effects of putative breast cancer predisposing genes, it is necessary to have access to a bank of cells of an appropriate phenotype, derived from such individuals. As the great majority of breast cancers are derived from luminal cells in the breast epithelium, it is these cells that must be acquired and established *in vitro* as the primary resource. In addition, however, there is accumulating evidence for a role of fibroblasts in both the modulation of mammary morphogenesis and tumor progression. In order to cover all possible mechanisms of action of predisposing genes it is therefore necessary to establish cultures of stromal cells and myoepithelial cells from the same patients.

A number of groups including our own have, over the past decade, developed methods whereby the cells from human breast epithelium, which include both luminal and myoepithelial types, can be cultured *in vitro* and cloned (6). However, we are the first group to have developed methods whereby the constituent cells of this epithelium can be separated and cultured as pure cell populations. Our initial studies utilised FACS and exclusively expressed surface antigens present on the different epithelial cell types to sort them. This method has given populations of very high purity (>98%) but in relatively low yields (<10⁵ cells/preparation). Such preparations have, however, enabled us to demonstrate that it is the myoepithelial cells, which de-differentiate in culture to give a simple basal epithelial phenotype, which rapidly come to dominate 'mixed' cultures derived from the intact epithelium. As such cells do not seem frequently to give rise directly to breast cancers, they must be separated in bulk from the luminal component if relevant culture systems are to be established from genetically pre-disposed individuals. In addition, methods have had to be developed for efficient conditional immortalisation of small numbers of cells (7) (described in more detail in Section 6). We have recently started to use the amphotropic system developed by Denise Galloway (8), which is based on the HPV 16, E6 and E7 genes for cell immortalisation.

We have been successful in obtaining breast tissue from a number of women with an increased familial risk of breast cancer and have established primary cultures from a high proportion of these.

(ii) Part 2 *In Situ* Data-Base:

Owing to the breast screening programmes, pathologists are seeing an increasing number of small tumors of which approximately 25% are ductal carcinoma *in situ*. This is providing more material for experimentation, but as these lesions have a good prognosis, long term follow up is required before any parameters measured in these tumors will be evaluable as predictors of behaviour. It is, therefore, essential that large series of retrospective cases are accumulated that can be used to correlate the results of future *in vitro* gene expression experiments with the *in vivo* pattern of expression and how it relates to the stage in tumor progression and parameters that can be measured directly in tumor samples. In addition it is clear that *in situ* breast cancer is itself an heterogeneous disease at the molecular level (9,10,11). To arrive at answers as to the relative importance of new genetic abnormalities it

will, consequently, be necessary to combine the data from many large centres that specialise in breast cancer. It can be predicted that pathologists will in the future be defining a molecular "bar-code" of *in situ* disease which will give predictive rather than prognostic information. It is, therefore, essential that large banks of early lesions are available to assess the relative importance of individual genetic abnormalities and the order in which they occur. By pooling material and data it will be possible to obviate the reporting of small series that are often misleading and remain unsubstantiated. In this context the objective of this part of the proposal is to identify a well characterized group of *in situ* cancers.

The Breast Diagnostic Unit of the Royal Marsden Hospital recruited its first patient in 1967. The objective was to offer a screening service to women who were perceived to have a high risk of breast cancer. The criteria used for defining a family history at that time were rather ill defined and thus all patients with a first or second degree relative affected were recorded. Clinical data, mammograms, and information on risk factors are recorded on the majority of the 30,000 patients seen since that time which includes over 600 cases with pure *in situ* carcinoma, according to the original pathology reports. The *in situ* cancers in this data set are a self selected population and thus not representative of a modern screening population; however, the material is valuable owing to the long median follow up and its use for molecular and immunohistochemical studies. A priority has been in this first two years to establish a separate data-base of the *in situ* cases and to review the pathology using modern criteria. This has been done in conjunction with a record of all the clinical data available relating to the macroscopic appearance of the lesions, diagnostic tests, treatment and follow-up (see APPENDIX for information now included on the data-base). During the pathology review we have identified representative paraffin blocks that contain material for future studies.

In the last year we have extended the data-base to include all cases of LCIS and DCIS from 1994-1996. This has provided a further 100 cases that we have reviewed for future pilot studies. We have recently received 3 year support for a study to carry out a detailed molecular analysis of some of these cases and in addition access to the data-base has provided the possibility of a collaboration (Drs R Houlston and M Stratton) starting a linkage study on families with LCIS, supported by the Cancer Research Campaign.

(6) BODY

(i) Familial Breast Cancer In Vitro

a) The Structure of the Cancer Family Clinic

Risk estimates are computed from the family structure. If the family is likely to be carrying BRCA1 (families with both breast and ovarian cancer or families with >4 cases at less than 50 years), the risks are computed from the Breast Cancer Linkage Consortium data. Risk figures for Li-Fraumeni and Li-Fraumeni-like families are computed where gene carriers have a 90% risk of breast cancer before 45 years. Risk figures for individuals in families unlikely to be due to BRCA1 or p53 are computed from the Claus study (12). Referrals are sent from surgeons, oncologists and mammography screening centres nationwide. We have had problems in receiving sterile specimens from other hospitals. This has resulted in the loss of many specimens due to bacterial or fungal contamination, presumably carried over from the pathology cut up area. However, in spite of these limitations we have managed to establish

cultures from 38 referred cases and have commenced immortalisation of 19, using the methods described below.

b) Material resources:

Prior to starting this project the genes (other than mutant p53) which pre-dispose to breast cancer in a familial context had not been identified. Even though the BRCA1 and BRCA2 genes have been identified the women in the UK coming to surgery do not have access to the test for mutations in this gene. This is unlikely to change in the next 6-9 months due to financial restrictions in the National Health Service. We are currently using samples from normal individuals at high risk of subsequent cancer, and affected women (>75% of cases are affected). This approach requires the processing of a relatively large number of samples so as to ensure that at least some samples are from *bona fide* carriers. These samples will then be tested retrospectively once the tests are routinely available. As can be seen from Table 1 in the Appendix we have received samples from 38 patients for this project.

c) Tissue preparation:

On receipt of the specimens they are subjected to routine pathology description and investigation for which Professor Gusterson is responsible. All patients at the Royal Marsden Hospital and referring hospitals give informed consent for all tissue removed at operation to be used for research purposes.

Samples for culture are processed as described previously (6). Briefly the breast tissue is chopped into a fine mince with scissors and the epithelial "organoids" prepared by progressive collagenase digestion, sedimentation and filtration. Primary epithelial cultures will be prepared by seeding 1,000 to 2,000 stroma-free organoids into 75 cm² plastic culture flasks in RPMI 1640 medium with 10% (v/v) fetal calf serum, 5 µg/ml hydrocortisone, 5 µg/ml of insulin and 100 ng/ml of cholera toxin plus penicillin and streptomycin. After 7 days, when the organoids have mobilised and spread to form near-confluent epithelial cultures cells are harvested by trypsinisation. Samples of all cell types in primary epithelial cultures are harvested and stored, in replicate, as frozen cell samples in liquid nitrogen. These can be retrieved and used at a later date for bulk cell preparation using the methods described. In this first year, only the Li Fraumeni patients have had a proven genetic phenotype and thus in the majority of cases we have not processed the tissue further.

d) Epithelial cell separation and immortalization:

Mixed epithelial cultures have been further processed in selected cases by MACS sorting on the basis of the exclusive expression of the epithelial membrane antigen by luminal cells and the expression of CD10 on myoepithelial cells as previously described (13). Using this type of methodology it is possible to produce in excess of 10⁷ cells. Purified populations of cells have been obtained where possible and stored for further analyses.

The following explains the technique used to establish immortalized cells. Having established a high titre amphotropic packaging line producing replication-disabled retrovirus that encodes the tsA58-U19 gene within the pZip(neo)SV(X)1 vector, we have used this to immortalize purified human mammary cells in the following manner. FACS sorted preparations of epithelial membrane antigen positive cells have been established in short-term clonal culture,

as described by (6). After selection for the neomycin resistance gene that forms part of the vector, a pure population of tsT-antigen expressing cells is obtained (7). Fibroblasts are also purified from the digested breast tissue and stored in liquid nitrogen. Although an SV40 based system has limitations in so far as effects of the viral gene are concerned these are minimised by the use of a temperature sensitive system. At this time it is the most efficient and controllable system available for reproducible immortalization of human cells. As stated above we have commenced the immortalization process on cultures from 19 patients/women. In the last six months we have also started to use an amphotropic system developed by Dr Galloway (8) that utilises the E6 and E7 genes of human papillomavirus type 16.

In the original proposal we set ourselves a number of tasks. Below is a summary of achievements measured against the objectives:

Objective:

Task 1, Separation and banking of epithelial and stromal cell types from breast tissue of predisposed individuals, Years 1-2:

- a. Breast tissue will be separated into component cell types using a combination immunomagnetic (epithelial) and selective digestion (stromal) techniques.
- b. Cultures will be assessed for relevant purity using flow cytometry of cell-type specific antigens and multiple immunofluorescence methods.
- c. Pure cultures will be banked in replicate in liquid nitrogen to await identification of specific predisposing genotype.

Achievement:

We have continued to make considerable progress in the culture work which is very labour intensive. We aimed to use years 1 and 2 to produce the primary cultures. In many preparations a,b and c have been achieved (see Table 1 in Appendix). We have had to train a new member of staff in this difficult technique so the success rate has been very good. In year 2 we proceeded with more specimens and commenced the immortalization of selected cultures where we had a very strong family history or knew the molecular phenotype. This has resulted in us establishing primary cells and commencing immortalisation of primary cells from four known p53 mutations (one is a splice donor site mutation and the others are mutations in codons 243, 248 and 273). The p53 mutations are being analysed in collaboration with Dr Eeles who is a co-applicant on the grant. We have primary cells and infected cells from two patients with known BRCA1 mutations, both of which are deletions in exon 11 (del 1294 and a four base pair deletion at 4184).

We have large numbers of cells that are in primary culture and at the stage of primary infection. One difficulty that we have is that we do not have our own facilities to carry our BRCA1 and BRCA2 screening on the affected cases. Also because the patients are being taken through the routine genetics clinic and counselling system we have to wait some months for the molecular analyses. In the UK there are major funding problems for screening of BRCA1 and BRCA2 mutations. It is therefore to be predicted that we will have to pay for the test on selected cases. In addition the analyses will only detect the mutation in 80% of

cases and in the other 20%, with strong linkage, the mutation can not be identified within the coding sequence of BRCA1. Over the next year we have, therefore made the strategic decision to concentrate on the cases where we know the mutation status and to move on to the other material when the mutation is confirmed. We have cells from three patients that appear to be BRCA2 families, in two of which the index cases were male breast cancers.

Task 2, Establishment of cell lines from specific genotypes, Year 3:

- a. Examples of high penetrance genotypes will be immortalized using retroviral gene transfer and studies initiated to characterise the cells.
- b. We will initiate our xenograft programme of tumours from BRCA1 and BRCA2 cases. This was not an objective in the original programme, but we will aim to do this for the reasons indicated below:

Estimated numbers of tumours available to us from different categories of patients at increased risk:

BRCA1 and BRCA2 Carriers

The percentage of breast cancer cases diagnosed at <50 years which are due to BRCA1 is estimated to be under 5%. It is conceivable that a high proportion (as high as 80% data from our clinic), of BRCA1 carriers would have bilateral mastectomy. There are 6000 cases of breast cancer per year diagnosed at <50 years in the UK, so 300/year may be carriers of a BRCA1 mutation. At the Royal Marsden Hospital 2,500 patients have been entered into the Tamoxifen prevention programme. The majority of these patients were selected for the trial because of their strong family history. The trial has now been underway since 1985 and some of the well women (46) are now developing breast cancers. These women are being tested for BRCA1 and BRCA2 and the material is available for research purposes.

Li Fraumeni and Li Fraumeni-like Families

We are the first centre in the UK to offer predictive TP53 gene testing and have established collaborations with seven other major UK genetics clinics who refer patients for testing and mastectomy samples from prophylactic operations. About five mastectomies/year would be obtained from these families.

Ataxia Telangiectasia

It has been estimated that up to 7% of breast cancer cases may be due to this gene. We estimate that about 10 prophylactic mastectomies/year would be in AT heterozygotes.

(ii) Task 3, Establish a data-base and tissue bank of *in situ* disease

Objective:

The cell biological resource produced by the technique described above will facilitate research by producing cell systems that can be utilised for analyses of genes involved in the multistep process of breast cancer. It will, however, be necessary to constantly return to the actual disease to assess the relevance of these findings. It is therefore the purpose of this part of the

proposal to establish a data-base of patients presenting with purely *in situ* breast cancers and epithelial atypias at the Royal Marsden Hospital since 1967.

Achievement:

We have identified over 700 cases recorded as *in situ* carcinoma that are to be considered for incorporation into the data set. In the Appendix a table of the information to be recorded on the data-base is provided together with examples of the Histopathology Review Form, the Patient Information Check List that has been used and an example of the patient data recorded. The following has been carried out and recorded on over 600 cases that have been reviewed so far and are on the data-base. A further 100 cases have had histology review, but are not on the database.

- a) The histology of all material on these cases has been reviewed by Professor Gusterson and information put on the data base from the Histopathology Review Form. The review form is identical to that used in the UK National Screening Programme. This form has, however, been recently amended to incorporate a new definition of DCIS and its grading (14). The grading system is based on that agreed by the European Pathologists Working Group and in addition includes a definition of atypical ductal hyperplasia using the criteria of Page (15).
- b) Tissue blocks that contain sufficient material have been identified and marked for future study. In particular data has been recorded to identify interesting cases where transitions could be defined from normal, through epithelial proliferation without atypia, to atypia, and *in situ* carcinoma.
- c) We have cut one unstained section and 10 unstained sections mounted on silane coated slides for future use. In addition, blocks have been identified that have sufficient material for microdissection of DNA from specific lesions.
- d) In 450 cases clinical information has been recorded for future clinico-pathological correlations. We have still to complete the 'flagging' of all cases so that registration of subsequent cancer in the case of the benign diseases, and of death can be recorded. This is being carried out in conjunction with the National Health Service Central Register and the Local Cancer Registries.
- e) Because patients coming to the Breast Diagnostic Unit were considered to be of high risk, they include many cases that appear to have a family history of breast cancer. It is, however, essential that proper family histories are taken. We have now carried out family histories on all cases of LCIS as part of another study (see later), and the data is being incorporated into the data-base. Within this data set there are cases of metachronous and synchronous bilateral disease. These have been recorded. Family histories on the DCIS cases have not been carried out.
- f) We have identified those cases of *in situ* cancer where it is difficult to establish the presence or absence of microinvasion, as these may be useful for future studies.

We have therefore made a considerable impact on our objectives, having almost completed the review and the data-base. We have histologically reviewed all of the

new cases 1993-1996 and identified cases suitable for pilot molecular studies. It is clear that pathologists have great difficulty in agreeing an objective criteria for diagnosing atypical lesions (16) and as can be seen below we have won funding to investigate this using molecular profiling.

- g) We have used the data-base to assess the best methods of grading of DCIS: This is based on a comparison of three proposed methods (14,17 and 18). The results have been submitted for publication.
- h) As stated below we are intending to use this material for molecular studies to address specific questions in relation to the variant phenotypes seen in *in situ* breast cancer using LOH analyses at specific loci. These studies are supported by other funding sources and will utilise the expertise that we already have in this area. We have used the data-base to carry out preliminary studies that have confirmed that the material is suitable for both LOH and CGH analyses (submitted for publication).

New Studies Using the Data-Base:

1. *The Role of Genetic Susceptibility in lobular carcinoma in situ (LCIS)* (R Houlston and M Stratton, in collaboration with B Gusterson)

LCIS confers an elevated risk of invasive cancer. Over the twenty-five years following diagnosis, approximately one-fifth of LCIS cases will develop invasive cancer. Many of these occur in young women, and as a result the relative risk of breast cancer in LCIS cases is high, of the order of 10. Invasive cancers are equally likely to occur in the contralateral breast as in the breast known to carry LCIS, consistent with the observation that the disease is frequently multicentric. This is in contrast to partially resected DCIS in which the invasive cancer usually develops in the same quadrant of the same breast as the DCIS. A proportion of LCIS cases also develop second primaries. 50% of invasive cancers developing upon a background of LCIS are lobular in histological type, the remainder being a mixture of ductal NOS, tubular and others.

The biological nature of LCIS and its relationship to invasive cancers is controversial. The multicentricity of the disease has led some authors to propose that it is a hyperplastic rather than a neoplastic process. Some authorities regard it solely as a risk indicator for invasive cancer or a morphological marker for the carcinogenic stimulus, implying that the cancer itself does not arise from the abnormal LCIS cells. An alternative view, which is generally accepted for DCIS, is that LCIS cells are intermediates in the progression to invasive cancer.

The aetiology of LCIS is also unclear. The preponderance of young premenopausal women with this disease is suggestive of a dependence upon endocrine factors. However, oophorectomised women have also developed LCIS and the disease is not restricted to premenopausal women. Moreover, dependence upon endocrine factors does not mean that the carcinogenic influence itself is endocrine mediated. The pattern of early age of onset and multicentricity invite consideration of a heritable susceptibility. This is supported by recent data from our laboratory which indicate that foci of LCIS are clonal (19). These results suggest that LCIS is a disease characterised by multiple low grade neoplasms, a pattern reminiscent of other heritable conditions such as familial polyposis coli or neurofibromatosis. However, there is no direct information concerning the familiarity of LCIS, or the risk of

invasive cancers in relatives of patients with LCIS. LCIS is not a notable feature of known breast cancer predisposition syndromes such as those due to the BRCA1 or p53 genes and therefore may be an indicator of a previously unrecognised, novel cancer predisposition syndrome in which the penetrance for invasive cancer is relatively low.

In this study the aim is to assess the risk of breast and other cancers in relatives of patients with LCIS. This work is funded by the Cancer Research Campaign and is using the cases of LCIS in the data-base as the index cases for both follow up to investigate the phenotype of the invasive tumours subsequent to the LCIS and the tumours arising in family members. This work has been approved by the Royal Marsden Hospital Ethics Committee.

2. *A molecular analysis of LCIS, DCIS and ADH*

The National Breast Cancer Screening Programme has resulted in a large increase in the proportion of breast lesions biopsied that produce diagnostic difficulties. This is reflected in the inconsistencies in diagnosis between pathologists in the National Quality Assurance Scheme. Many of these "difficult" lesions are of unknown biological significance. Removal of lesions could result in some instances of overdiagnosis, resulting in an apparent decrease in mortality statistics and an increase in incidence of cancer. Under diagnosis of malignancy will result in the converse. Recent molecular advances may facilitate rapid analyses of these diagnostic dilemmas, but firstly it is important to establish the molecular profile of the malignant phenotype. This proposal builds on a unique data-base of over 450 *in situ* breast carcinomas and atypical lesions. The material provides a resource in which to establish the molecular phenotype of specific morphological entities and borderline diagnoses. Recently developed methods will enable detailed allelotyping and in parallel comparative genomic hybridisation (CGH) analysis, to identify chromosome gains and losses, of these lesions microdissected from paraffin embedded material. Combining these molecular methods with routine pathology we aim to establish, within a three year project, a molecular profile that will enable an objective assessment of difficult breast lesions and identify consistent areas of gene amplification and chromosome loss that will facilitate future studies to identify genes involved in the early stages of tumour progression.

a) *Ductal carcinoma in situ:*

Ductal carcinoma *in situ* (DCIS) accounts for approx. 5% of symptomatic and approximately 20% of screen detected cancers. It is thus a lesion which is of increasing clinical significance. There is no consensus on how to treat woman with DCIS or, atypical ductal hyperplasia (ADH). This reflects the fact that little is known about the natural history or the molecular biology of these lesions and how they relate to the invasive carcinoma that arise in the same women. There have been many recent attempts to classify DCIS, using features such as nuclear grade, morphological growth pattern and necrosis. A recent study has shown that the current classification as advised by the National Coordinating Group for Breast Screening Pathology is the most consistent in predicting recurrence of invasive or *in situ* disease.

The classification referred to above is not ideal for the reporting of breast lesions as all cases with cytological atypia that do not conform to the strict criteria of atypical ductal hyperplasia are classified as benign. This is a good pragmatic approach to produce a consistent classification, but the true malignant potential of the lesions that are cytologically atypical must be addressed. As the lesions are excised, this can not be done by follow up

studies, but only by analysing their molecular profile in comparison with lesions that are definitely malignant.

There are other important questions that need to be addressed. The relationship between the small cell variants of DCIS (cribriform and micropapillary) and LCIS is worthy of investigation. In addition there are a significant number of small cell variants of DCIS that have a solid growth pattern and are very uniform with a morphology similar to the cells seen in LCIS. These lesions are the subject of considerable disagreement in classification between pathologists. Another important issue is the relationship of epithelial hyperplasia without atypia, to ADH and DCIS.

b) Lobular carcinoma in situ

Lobular carcinoma *in situ* (LCIS) accounts for 0.5% of symptomatic and 1% of screen detected cancers. An estimated 15-20% of women with LCIS develop invasive carcinoma, frequently of the ductal type, and a further 10-15% will develop breast cancer of the other breast. There is no real consensus on how to treat woman with LCIS or the putative precursor lesion, atypical lobular hyperplasia (ALH). This reflects the fact that little is known about the natural history or the molecular biology of these lesions and how they relate to the invasive carcinoma that arise in the same women

Limited findings suggest that lobular lesions may arise through different molecular mechanisms to those described as ductal, which is in keeping with the different clinical and morphological picture. LOH analysis carried out at the Institute/RMHT on LCIS indicated some genetic differences to DCIS (19). In addition, limited allelotyping of DCIS (20) and of atypical ductal hyperplasia (ADH) indicated the clonal nature of ADH as defined by the National Screening Programme and the similarity in phenotype between comedo DCIS and an invasive component in the same tumour. Loss at 16q22 has recently been shown to involve the cellular adhesion molecule, E-cadherin, specifically in lobular cancer (21).

We have identified a large number of cases in the data-base where DCIS, ADH and epithelial hyperplasia without atypia occur in the same specimen. In addition we have identified cases where DCIS is associated with LCIS (see Appendix).

Methods:

Molecular genetic analysis.

a) LOH analysis. A set of primers covering 220 loci at intervals of approximately 13centiMorgans is available to use for this study. Initially a set of 40 or 80 evenly distributed markers, which yield small products, will be used in the manner previously described by Lakhani et al. (19). As the amount of material dissected from hyperplastic and in situ lesions limits the analysis to 4-6 markers per lesion, samples with large numbers of in situ lesion have been selected and will be analysed with different markers. Although heterogeneity between individual lesions is expected, consistent, implying significant, regions of allele loss between samples may emerge. The allelotype analysis will be carried out in collaboration with Dr. Sunil Lakhani (formerly at the Royal Marsden Hospital and now at University College, London) and Dr Mike Stratton (Reader in Molecular Genetics at the Institute of Cancer Research), who have extensive experience in this area. The amount of material from the associated invasive lesions should not be limiting and a detailed allelotype of

these will be determined. The aim is to determine consistent regions of allele loss and to produce the most comprehensive profile of the allelotype of the lesions as possible.

b) CGH analysis. The approach of CGH will screen the whole genome for gains, losses and amplification of genomic material and is therefore complementary, and may provide supplementary information to, the LOH analysis. CGH was originally described using high molecular weight DNA from cell lines and fresh material. We have previously used CGH to identify gains and losses of material in rhabdomyosarcoma (22,23) and, using cell lines with known amplicons, shown that it is possible to detect copy number changes affecting in the region of 2-10 megabases of DNA (24). Combining this approach with the polymerase chain reaction using degenerate oligonucleotide primers (DOP-PCR) sufficient representative DNA can be generated to enable analysis of archival material and small regions of specimens in a way not previously possible. The DNA produced and normal reference DNA are differentially labelled with fluorochromes and hybridised to normal metaphase chromosomes under suppression conditions with unlabelled Cot1 DNA. A fluorescence ratio outside the normal range at a particular chromosome location is indicative of a copy number change in that region. The feasibility of using paraffin material in this way was first demonstrated in late 1993 (25). We have recently established this approach and in the appendix is a demonstration of using paraffin embedded material. The copy number changes of specific chromosomes indicated by CGH would be confirmed by hybridising probes to paraffin embedded sections. We have previously performed this type of analysis.

A comprehensive molecular genetic profile of approximately 100 dissected samples would be carried out during a 3 year period. This data would allow the issues set out in the aims to be addressed, including possible correlations with clinical data held in the database. We have identified a large number of cases in the data-base where DCIS, ADH and epithelial hyperplasia without atypia occur in the same specimen. In addition we have identified 10 cases where LCIS was followed by infiltrating ductal carcinoma and 10 cases where DCIS is associated with LCIS. It will be of particular importance to investigate whether there is evidence at the molecular level for the ductal carcinoma having evolved from the 'precursor' LCIS and the relationship between LCIS and DCIS in the same breast. This can be done by following the molecular profile of the lesions using both CGH and allelotyping analyses.

(7) CONCLUSIONS:

We have made significant progress in the last year in meeting our objectives and targets. In relation to the familial breast cancer work the major change that we have had to consider is the cloning of BRCA2. This has meant that we have decided to concentrate our immortalisation work on those cases known to have mutations. Primary cultures have been infected from two BRCA1 and four Li Fraumeni cases.

In relation to the *in situ* data-base: Progress has been faster than we predicted and we have the data recorded on all available cases up until 1994. Cases from 1994-1996 have been reviewed, but need to be entered on the data base. by August 1996. In addition we will have most of the sections cut by that time. We have had to make an assessment of which blocks are the best to use for staining and molecular studies. This is due to the fact that the review has shown that in many cases the diagnosis was based on a small focus of abnormal proliferation that is no longer in the block. This is important for any clinical correlations and indicates the problems of sampling bias that can be introduced into certain studies using this material,

where clinical parameters are used as an end-point. For molecular correlates of morphology, however, the data is very valuable. Molecular genetic studies are underway with this material.

NOTE:

As was identified by the referees at the time of review of this project there will be a need to consider continuation of funding to maintain the data-base and the pathology material collected. In addition it is unlikely that the cell lines will be characterised within the next year, as the immortalisation process can take a year for the cells to emerge from crisis. Also we are likely to have in culture cells from BRCA2 and ?BRCA3 and these will be coming through later. The expansion of the programme to the tumours is a logical extension as we have cases coming through from the Tamoxifen prevention programme. It would be useful to me if the reviewer would indicate if there would be support to extend funding for this programme to enable us to continue with the biology project and to have limited support for the maintenance of the data base.

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APPENDIX I

TABLE 1

CASE	STATUS	FROZEN TISSUE	PRIMARY CULTURE	SEPARATED CELLS				INFECTED CELLS			
				LUMS	MYOS	FIB	KERAT	LUMS	MYOS	FIB	KERAT
1	BrCa1 +ve	N	Y	Y	Y	Y	Y	Y	Y	Y	N
2	BrCa1 +ve	Y	Y	Y	Y	Y	N	Y	N	N	N
3	Li Fraumeni	N	Y	N	N	Y	Y	N	N	N	N
4	Li Fraumeni	N	Y	N	Y	Y	Y	N	N	N	N
5	Li Fraumeni	Y	Y	N	N	Y	N	N	N	N	N
6	Li Fraumeni	Y	Y	N	N	Y	Y	N	Y	N	N
7	Li Fraumeni	Y	Y	Y	N	Y	Y	Y	N	N	N
8	Li Fraumeni	Y	Y	Y	Y	Y	N	N	Y	Y	N
9	Li Fraumeni	Y	Y	N	N	Y	N	Y	Y	Y	Y
10	Li Fraumeni	N	Y	N	Y	Y	Y	N	N	N	N
11	RH	Y	N	Y	Y	Y	N	Y	Y	Y	N
12	RH	Y	Y	N	N	Y	N	N	N	N	N
13	RH	Y	Y	N	N	Y	N	N	N	N	N
14	RH	Y	N	N	N	N	N	Y	N	Y	N
15	RH	Y	Y	Y	N	Y	N	Y	Y	Y	N
16	RH	Y	Y	N	Y	Y	N	N	Y	Y	N
17	RH	Y	Y	N	N	Y	N	N	N	N	N
18	RH	Y	N	N	N	N	N	N	N	N	N
19	RH	Y	Y	N	N	Y	N	N	Y	Y	N
20	RH	Y	N	Y	Y	Y	N	N	N	N	N
21	RH	Y	Y	Y	Y	N	N	N	N	N	N
22	RH	Y	Y	Y	N	N	N	Y	N	N	N
23	RH	Y	Y	Y	N	Y	N	N	N	Y	N
24	RH	Y	Y	N	N	Y	N	N	N	Y	N
25	RH	Y	N	N	N	N	N	N	N	N	N
26	RH	N	Y	N	N	Y	N	N	N	N	N
27	RH	Y	N	N	N	N	N	Y	N	Y	N
28	RH	N	N	N	N	Y	N	Y	N	Y	N
29	RH	Y	N	Y	Y	Y	N	Y	N	N	N

TABLE 1

CASE	STATUS	FROZEN TISSUE	PRIMARY CULTURE	SEPARATED CELLS				INFECTED CELLS			
				LUMS	MYOS	FIB	KERAT	LUMS	MYOS	FIB	KERAT
30 FH		N	Y	N	N	N	N	N	N	N	N
31 FH		N	N	Y	Y	Y	N	N	N	N	N
32 FH		Y	Y	N	N	N	N	N	N	N	N
33 FH		Y	Y	N	N	Y	N	N	N	N	N
34 FH		Y	N	Y	Y	Y	N	Y	N	Y	N
35 FH		N	Y	Y	Y	N	N	Y	Y	Y	N
36 FH		N	N	N	N	Y	N	N	N	Y	N
37 FH		Y	N	Y	Y	Y	N	Y	N	N	N
38 KF		Y	Y	N	N	N	N	N	N	N	N

KEY: FH = Family History
 KF = Klienfelter's Syndrome
 Y = Material Frozen
 N = No Material Frozen

BREAST SCREENING HISTOPATHOLOGY

Surname Forenames Date of Birth
 Screening no Hospital no Report no

Side ☐ Right ☐ Left Histological Calcification ☐ Absent ☐ Benign ☐ Malignant

Specimen radiograph seen? ☐ Yes ☐ No Mammographic abnormality present in specimen? ☐ Yes ☐ No ☐ Unsure

Specimen type ☐ Localisation biopsy ☐ Open biopsy ☐ Segmental excision ☐ Mastectomy ☐ Wide bore needle core

Specimen size (excluding mastectomies and needle core biopsies) x . x mm.

HISTOLOGICAL DIAGNOSIS ☐ NORMAL ☐ BENIGN ☐ MALIGNANT

For BENIGN lesions please tick the lesions present

- ☐ Fibroadenoma ☐ 'Fibrocystic change'
- ☐ Papilloma ☐ Single ☐ Solitary cyst
- ☐ Multiple ☐ Periductal mastitis/duct ectasia
- ☐ Complex sclerosing lesion/radial scar ☐ Sclerosing adenosis
- ☐ Other (please specify)

EPITHELIAL PROLIFERATION

- ☐ Not present ☐ Present with atypia ('ductal')
- ☐ Present without atypia ☐ Present with atypia (lobular)

For MALIGNANT lesions please tick any of the following present

NON-INVASIVE

- ☐ Lobular ☐ Paget's disease ☐ Ductal Subtype
- ☐ Cribriform
- ☐ Solid
- ☐ Papillary
- ☐ Micropapillary
- ☐ Comedo

MICROINVASION

- ☐ Not present ☐ Possible ☐ Present

INVASIVE

- ☐ 'Ductal' (Not otherwise specified) ☐ Tubular or cribriform carcinoma
- ☐ Medullary carcinoma ☐ Mucoid carcinoma
- ☐ Lobular carcinoma
- ☐ Other primary carcinoma (please specify)
- ☐ Other malignant tumour (please specify)

MAXIMUM DIAMETER (invasive component) mm (in-situ) mm

AXILLARY NODES

OTHER NODES Site ☐ Not Present Number positive Total Number

..... ☐ Not Present Number positive Total Number

EXCISION

☐ Reaches Margin ☐ Does not reach margin (Distance mm) ☐ Uncertain

GRADE

☐ I ☐ II ☐ III ☐ Not assessable

DISEASE EXTENT

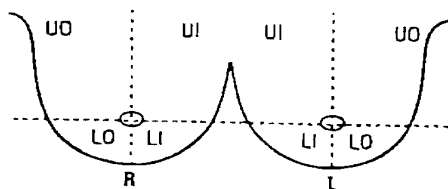
☐ Localised ☐ Diffuse single quadrant ☐ Multiquadrant ☐ Not assessable

VASCULAR INVASION

☐ Present ☐ Not seen

SITE (Optional)

COMMENTS/ADDITIONAL INFORMATION



PATHOLOGIST

☐ Case for review?

DATE

INFORMATION CHECKLIST
(Information search in patient's records etc.)

1	SURNAME DATE OF BIRTH	HOSPITAL NUMBER
2	ASYMPTOMATIC SYMPTOMATIC (tick)	
3	FAMILY HISTORY	
	BREAST CANCER	RELATIONSHIP AGE AT DIAGNOSIS
	OTHER CANCER	RELATIONSHIP TYPE AGE
4	HOSPITAL & DATE OF OPERATION	
	1	2
5	BREAST/SIDE	
6	SITE IN BREAST	
7	OPERATION PERFORMED	
8	CANCER OR BENIGN DISEASE TYPE	
9	SPECIMEN RADIOG./ABNORM. PRESENT	
10	RECURRENCE	
11	BILATERAL	
12	SURGERY	
	1	2
	3	
	RADIOTHERAPY (DATES) CHEMOTHERAPY (TYPE) HORMONE (TYPE)	
13	OTHER CANCER TYPE	DATE PREVIOUS SUBSEQUENT
14	OTHER CANCER TREATMENT	
	SURGERY RADIOTHERAPY (DATES) CHEMOTHERAPY (TYPE) HORMONE (TYPE)	
15	METASTSES SITE(S)	DATE(S) DIAGNOSED
16	LAST OPA DATE	HOSPITAL DATE FROM
17	DEATH DETAILS DATE	REG. CAUSE OF DEATH
18	COMMENTS (any other relevant information)	

A

-----,MISS-----

Date of first report 6.2.81

GENDER : sex : female

PATHOLOGY

6.2.81 Report : Pathology Number = --/- ; side : right; DCIS; foci : not stated; Comment = papillary intraduct ca.

2.12.81 Report : Pathology Number = --/- ; side : right; Recurrence; DCIS foci : not stated

21.8.85 Report : Pathology Number = --/- ; side : right; Recurrence; DCIS Invasive; foci : not stated

Reviewed : 18.9.92 Path Number(s) selected = --/- ; --/- BLOCKS 1 2

SLIDES : 8.9.93 slides cut

LAST UPDATE (SLIDE AND BLOCK LOCATION) : 19.8.92 Comment = 1985: 2999/85
intraduct and infiltrating papillary
ca. Slides not in FR Path. - out to
B.G.; slides location : AW; block
location : FR
4.9.92 slides location : BG; block
location : FR
18.9.92 slides location : FR; block
location : FR

-----,MISS-----

Date of first report 6.2.81

Family History : Family History of Breast Cancer; Mother; Age at diagnosis = NK

Pathology Details : Date of Birth = 6.2.24; Pathology reference number(s) = --/81; Date of Report = 6.2.81; Slides reviewed; Slides selected; Symptom status : symptomatic; side : Right; site : Other; specify = CENTRAL; Histological calcification : NK; Specimen type : open biopsy; Specimen size (excl mastectomy & needle core biopsy) : known; Largest diameter (mm) = 65; Second largest diameter (mm) = 30; Smallest diameter (mm) = 15; Size of second specimen : Not applicable; Histological diagnosis : Abnormal; malignant; Epithelial Proliferation : not present; malignant type : non-invasive; ductal; cribriform; Papillary; micropapillary; microinvasion : not present; Axillary nodes : not present; Other nodes : not present; Excision : NK; Grade : not assessable; disease extent : diffuse single quadrant; vascular invasion : NK

BILATERAL CASES

TOTAL NUMBER OF PATIENTS WITH IN-SITU CARCINOMA OF BREAST
IDENTIFIED TO DATE: 501

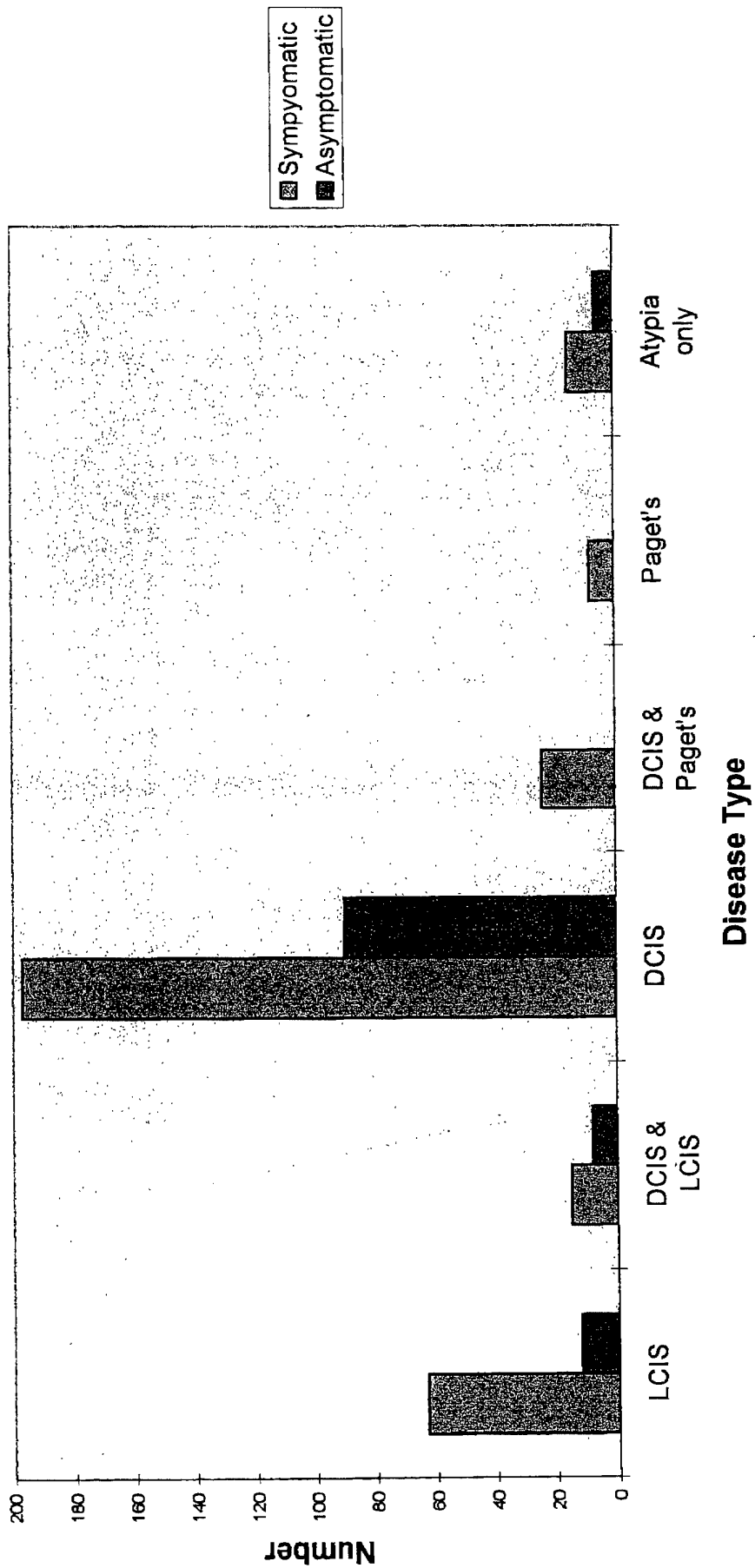
TOTAL PATIENTS WITH BILATERAL CA. BREAST: 73

(BILATERAL includes:

- 1 bilateral in-situ carcinoma of the breast
- 2 in-situ carcinoma in one breast with invasive carcinoma
in the contralateral breast)

SYNCHRONOUS	METACHRONOUS	TOTAL
31	42	73

Symptom Status at Presentation by Disease Type



TOTAL No. BY DISEASE TYPE

DCIS	305
LCIS	80
DCIS + LCIS	26
PAGET'S	8
DCIS + PAGET'S	25
ATYPIA	21
TOTAL	<u>465</u>

CASES WITH ATYPIA IN ASSOCIATION WITH OTHER FEATURES

	NONE	DUCTAL/ DUCTAL ATYPIA	LOBULAR/ LOBULAR ATYPIA	BOTH
DCIS	177	117	4	7
LCIS	23	7	37	13
DCIS + LCIS	7	7	3	9
DCIS + PAGET'S	23	1	1	-
ATYPIA		13	2	6

RECURRENCE OF *IN SITU* CARCINOMA

	<i>IN SITU</i>	INVASIVE	BOTH
DCIS	50	4	19
LCIS	16	2	1
DCIS + LCIS	2	1	1